Vascular defect beyond the endothelium in type II diabetic patients with overt nephropathy and moderate renal insufficiency

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There is a paucity of data on the effects of overt nephropathy and moderate renal impairment on endothelial function in diabetic patients. A total of 26 type II diabetic (DM) patients with nephropathy (DMN+) (mean±s.d. age: 63.7±6.3 years), 32 diabetic patients without nephropathy (DMN−) (59.4±10.1 years), and 52 non-diabetic subjects (54.9±8.2 years) were recruited. High-resolution ultrasound scan was used to measure carotid intima media thickness (IMT) and flow-mediated dilation (FMD) of the brachial artery. Endothelium-independent dilation was determined by maximal vascular dilation after sublingual nitroglycerine (glyceryl trinitrate (GTN)-induced dilation). The mean carotid IMT increased progressively from non-DM to DMN− to DMN+ groups (0.74±0.23 vs 0.80±0.25 vs 1.03±0.38 mm; $P=0.001$ for trend) whereas FMD- (4.3±2.5 vs 3.9±1.7 vs 1.9±2.0%, $P<0.001$ for trend) and GTN-induced dilation (14.7±4.0 vs 14.5±3.9 vs 10.3±3.2%; $P<0.001$ for trend) declined in an opposite manner. On multivariate analysis, age ($\beta=0.257$, $P=0.009$), glomerular filtration rate ($\beta=-0.364$, $P<0.001$), and smoking ($\beta=0.25$, $P=0.013$) were independently associated with carotid IMT ($F=15.76$, $R^2=0.340$, $P<0.001$). After adjustment for baseline brachial arterial diameter, history of smoking ($\beta=-0.039$, $P<0.001$), fasting plasma glucose ($\beta=-0.033$, $P=0.002$), and total cholesterol ($\beta=-0.023$, $P=0.024$) were independently associated with vessel diameter after FMD ($F=2446.5$, $R^2=0.992$, $P<0.001$); whereas age ($\beta=-0.069$, $P=0.001$) and urinary albumin excretion ($\beta=-0.048$, $P=0.018$) were independently associated with vessel diameter after GTN ($F=851.6$, $R^2=0.967$, $P<0.001$). Type II diabetic patients with overt nephropathy and moderate renal impairment had both structural and functional vascular abnormalities beyond the endothelium.

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The development of micro- and macroalbuminuria significantly escalates the risk of cardiovascular morbidity and premature mortality in type II diabetic (DM) patients[1–4] including Chinese.[5,6] There is growing evidence supporting that dysfunction of the vascular endothelium accounts for the propensity for atherosclerosis in this high-risk patient population. Flow-mediated dilation (FMD) as assessed by high-resolution ultrasound has been firmly established as a surrogate marker of generalized atherosclerosis.[7,8] Numerous studies have shown close association between impaired FMD and traditional cardiovascular risk factors[9] including type I and type II diabetes.[10,11] Furthermore, FMD is predictive of coronary events[12] and the severity of FMD impairment correlates with that of coronary heart disease.[13] More recently, chronic kidney disease has been found to be an independent predictor for cardiovascular events[14,15] especially in diabetic patients.[16] Whereas several studies have shown associations between microalbuminuria and endothelial dysfunction in both type I and type II diabetes,[7,17,18] there are limited data on the effects of overt diabetic nephropathy and renal insufficiency on vascular function in type II diabetes.

In this case–control cohort analysis, we examined the effects of moderate renal insufficiency and albuminuria on endothelium-dependent dilation and endothelium-independent dilation in type II diabetic patients and non-diabetic control subjects. We also examined the structural abnormalities of conduit vessels as reflected by common carotid intima-media thickness (IMT), another established marker of cardiovascular diseases.[19–21]

RESULTS

Among the three groups, patients in the DMN+ group were older, more likely to be smokers, had higher waist circumference, body mass index, systolic blood pressure, worse glucose, and lipid control as well as higher albuminuria and lower glomerular filtration rate (GFR) compared to the DMN− and non-DM group (Table 1). The mean carotid IMT increased progressively from non-DM to DMN− to DMN+ groups (0.74±0.23 vs 0.8±0.25 vs 1.03±0.38 mm;

$P=0.001$ for trend). FMD (4.3 ± 2.5 vs 3.9 ± 1.7 vs $1.9\pm2.0\%$, $P<0.001$ for trend) and endothelium-independent dilation (14.7 ± 4.0 vs 14.5 ± 3.9 vs $10.3\pm3.2\%$; $P<0.001$ for trend) declined in an opposite manner (Figure 1). The baseline brachial artery diameter also increased progressively from non-DM to DMN− to DMN+ groups (4.06 ± 0.74 vs 4.15 ± 0.62 vs 4.85 ± 0.68 mm, $P<0.001$ for trend). Peak systolic velocity did not differ among the three groups both at baseline (72.4 ± 5.0 vs 69.4 ± 4.8 vs 72.1 ± 3.9 cm/s $P=0.406$ for trend) and post-deflation of cuff (123.9 ± 15.7 vs 121.9 ± 12.6 vs 116.0 ± 12.4 cm/s. $P=0.073$ for trend). Vessel diameter after FMD showed progressive decrease from non-DM to DMN− to DMN+ groups (4.44 ± 0.18 vs 4.43 ± 0.18 vs 4.35 ± 0.19 mm $P<0.001$ for trend) after adjustment for baseline vessel diameter. Vessel diameter after glyceryl trinitrate (GTN) showed progressive decrease from non-DM to DMN− to DMN+ groups (4.86 ± 0.31 vs 4.88 ± 0.31 vs $4.73\ \pm0.16$ mm; $P<0.001$ for trend) after adjustment for baseline vessel diameter.

Multiple stepwise linear regression analysis was performed using age, sex, smoking (current and ex-smoker), blood pressure, body mass index, waist circumference, fasting plasma glucose, glycated hemoglobin, total cholesterol (TC), high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, GFR, and urinary albumin excretion (UAE) as dependent variables to identify independent predictors for IMT in the entire cohort of 110 control and diabetic subjects. The same set of independent variables together with baseline brachial artery diameter was used to identify independent predictors of vessel diameter after FMD and GTN.

Age ($\beta=0.257$, $P=0.009$), GFR ($\beta=-0.364$, $P<0.001$), and smoking ($\beta=0.25$, $P=0.013$) were independently associated with carotid IMT ($F=15.76$, $R^2=0.340$, $P<0.001$). Baseline brachial artery diameter ($\beta=1.007$, $P<0.001$), smoking ($\beta=-0.039$, $P<0.001$), fasting plasma glucose ($\beta=-0.033$, $P=0.002$), TC ($\beta=-0.023$, $P=0.024$) were independently associated with vessel diameter after FMD ($F=2446.5$, $R^2=0.992$, $P<0.001$). Baseline brachial artery diameter ($\beta=1.013$, $P<0.001$), age ($\beta=-0.069$, $P=0.001$), and UAE ($\beta=-0.048$, $P=0.018$) were independently associated with vessel diameter after GTN ($F=851.6$, $R^2=0.967$, $P<0.001$). Association between UAE and GTN-induced dilation is shown in Figure 2.

DISCUSSION

The major findings of this study are that in type II diabetic patients, the presence of overt nephropathy and moderate renal insufficiency was significantly associated with reduced endothelium-dependent dilation and endothelium-independent dilation as reflected by reduced response of vascular smooth muscle cells (VSMC) to nitric oxide (NO)

Table 1 | Comparison of baseline clinical and biochemical characteristics of Chinese type II diabetic patients with (DMN+) and without overt nephropathy and renal insufficiency (DMN−) as well as control subjects

Parameters	DMN+	DMN−	Control subjects	*P*-value (ANOVA)
Number	26	32	52	—
Age (years)	63.7 (6.3)	59.4 (10.1)	54.9 (8.2)	<0.001
Duration of diabetes (years)	16.7	6.7	NA	<0.001
Sex (M:F)	9:17	20:12	32:20	0.050
Smoker (ex and current)	57.7%	18.8%	19.2%	0.001
Body mass index (kg/m^2)	25.3 (2.6)	25.1 (3.4)	23.5 (2.5)	0.012
Waist circumference (cm)	93.6 (9.3)	86.0 (8.4)	82.8 (9.7)	<0.001
Systolic BP (mm Hg)	127 (19)	138 (20)	126 (20)	0.043
Diastolic BP (mm Hg)	85 (9)	79 (14)	83 (10)	0.174
FPG (mmol/l)	7.2 (2.0)	6.4 (1.7)	5.1 (0.4)	<0.001
HbA_{1c} (%)	7.3 (1.5)	6.8 (1.4)	5.5 (1.2)	<0.001
TC (mmol/l)	5.0 (1.3)	5.0 (0.8)	5.5 (0.9)	0.046
TG (mmol/l)	2.12 (1.63)	1.34 (0.63)	1.40 (0.68)	0.002
HDL-C (mmol/l)	1.14 (0.26)	1.39 (0.30)	1.48 (0.34)	0.001
LDL-C (mmol/l)	3.0 (0.9)	2.9 (0.8)	3.3 (0.9)	<0.001
UAE (mg/24 h)[a]	384 (4.8)	11.7 (2.9)	6 (2.3)	<0.001
Plasma creatinine (μmol/l)	299.5 (141.8)	75.5 (15.7)	72.3 (20.5)	<0.001
GFR (ml/min/1.72 m^2)	22.6 (11.5)	83.8 (16.1)	92.6 (21.2)	<0.001
Drug treatment				
Insulin therapy alone	77%	3.1%	NA	—
Insulin and oral drugs	0%	12.5%	NA	—
Oral drugs only	23%	84.4%	NA	—
BP-lowering drugs	96%	53.1%	NA	—
Brachial artery diameter (mm)	4.85 (0.68)	4.15 (0.62)	4.06 (0.74)	<0.001

HbA_{1c}, Glycated haemoglobin; FPG, Fasting plasma glucose; BP, blood pressure; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; UAE, urinary albumin excretion; GFR, glomerular filtration rate (estimated by Modification of Diet in Renal Disease Study formula).
Mean (s.d.).
[a]Express as geometric mean (antilog s.d.). DMN, diabetic patients with nephropathy; ANOVA, analysis of variance; M, male; F, female.

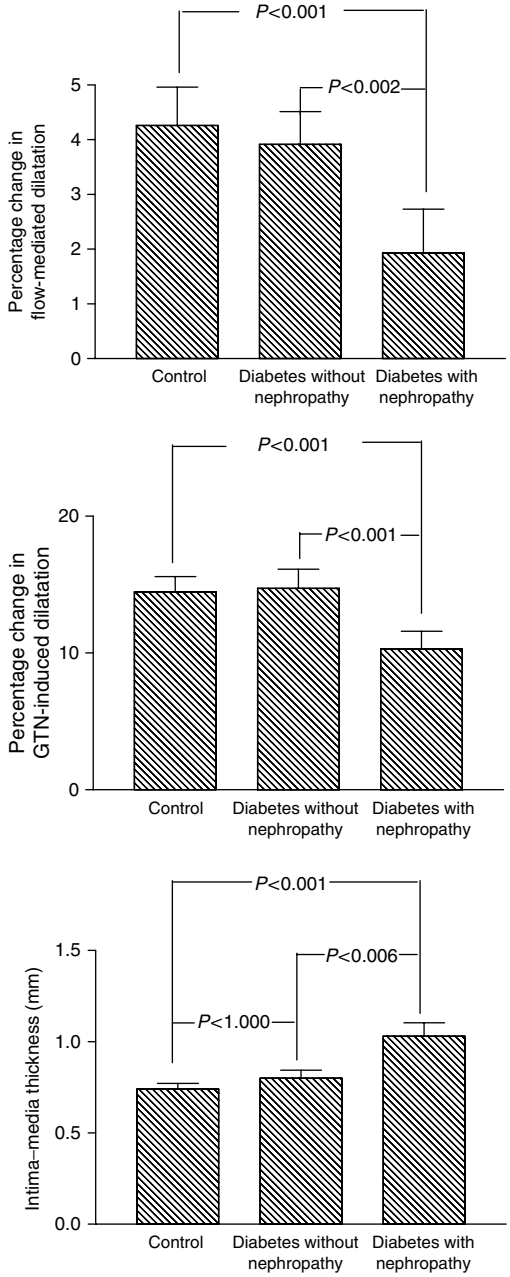

Figure 1 | Comparisons of vascular structure and function in control subjects and diabetic patients with and without overt nephropathy and renal insufficiency (panel 1: FMD of brachial artery; panel 2: GTN-induced dilation of brachial artery; panel 3: IMT, all data are shown as mean ± s.d.).

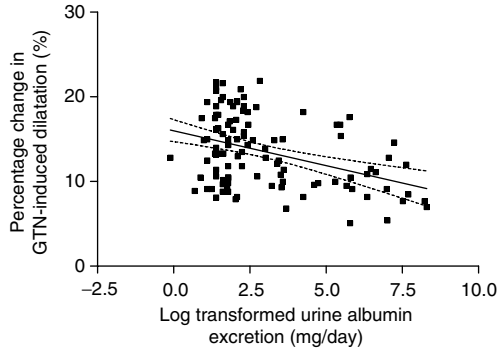

Figure 2 | The association between GTN-induced dilation of brachial artery and urinary albumin excretion in Chinese patients with varying degree of albuminuria and glycemia. The regression line (95% CI) is also shown with a correlation coefficient of −0.398 ($P < 0.001$).

administration. Our findings suggest that the major functional abnormality of diabetic vasculature, upon development of overt nephropathy, include decreased flow-induced endothelial NO production and reduced VSMC responsiveness to NO. In our study, diabetic patients with overt nephropathy also had greater increase in carotid IMT than diabetic patients without nephropathy and control subjects. Taken together, reduced NO production, dysfunction of VSMC, and increased IMT may contribute to the markedly escalated cardiovascular risk in patients with diabetic kidney disease.

Functional defects of diabetic vasculature in nephropathy

In the functional assessment of brachial artery using high-resolution ultrasound, FMD induced by reactive hyperemia has been known to be endothelium-dependent mediated predominantly by NO production from the endothelium.[22] In contrast, the vasodilator response to GTN is an endothelium-independent mechanism which reflects VSMC response to NO. The interpretation of FMD assumes normal VSMC response to NO. In our study, FMD and GTN-induced dilation were reduced to similar extent in diabetic patients with nephropathy suggesting that reduced NO production and reduced VSMC responsiveness to NO are both important in this group of patients. Previous studies have shown that in patients with type II diabetes, endothelial function of both forearm resistance vessels and conduit vessels was impaired whereas VSMC function was unaffected. These studies, however, were performed mainly in diabetic patients with normoalbuminuria or microalbuminuria.[23,24] In contrast, we have included patients with a wide range of metabolic control and renal function, thus despite our relatively small sample size, we were able to show the differential effects of these parameters on FMD and GTN-induced dilation.

In our study, after adjustment for baseline vessel diameter, smoking, plasma glucose, and TC were identified as major determinants of FMD, whereas GTN-induced dilation was independently predicted by age and albuminuria. In a previous case–control study involving diabetic patients without overt nephropathy and non-diabetic subjects, Stehouwer *et al.*[25] reported that FMD was impaired in patients with microalbuminuria whereas GTN-induced vasodilation was unaffected. Although we did not find an independent correlation between microalbuminuria and FMD, such relationship might be confounded by effects owing to smoking, plasma glucose, and TC which were also associated with albuminuria. On the other hand, despite controlling for

these conventional risk factors, albuminuria remained an important determinant for GTN-induced dilation. Since these changes were predominantly observed in patients with overt nephropathy and renal impairment, it is plausible that metabolic changes associated with chronic kidney disease might contribute to these associations. In this regard, abnormal bone metabolism, increased oxidative stress, and inflammation owing to retained urotoxins have all been suggested to accelerate vascular calcification and cause vascular stiffness in patients with chronic kidney disease, which can be further aggravated in the presence of diabetes.[15]

Carotid IMT in diabetic nephropathy

Increased IMT of the common carotid artery is a well-recognized surrogate marker of early atherogenic changes of the vasculature.[26] In type II diabetic patients with normoalbuminuria, carotid IMT has been shown to be increased[27,28] compared to non-diabetic subjects. While we did not observe significant differences in IMT between diabetic patients without nephropathy and control subjects, patients with diabetic nephropathy had marked increase in IMT. On multivariate analysis, smoking, GFR, and age were independent determinants of IMT, thus lending support to the adverse effects of the metabolic milieu of chronic kidney disease on the progression of atherosclerosis with involvement of both vascular structure and function.

Potential mechanisms for vascular defects in diabetic nephropathy

The mechanisms underlying VSMC and endothelial dysfunction in diabetic patients are complex, involving interactions between metabolic, hemodynamic, inflammatory, and growth factors. Besides, glucose-dependent changes such as increased formation of advanced glycation endproducts and activation of protein kinase C can further exacerbate these vascular changes.[29,30] In particular, the increased formation of glycation endproducts can impair the diffusion of NO across the basement membrane from the endothelium to VSMC. Alternatively, glycation endproduct accumulation may diminish the contractile ability of VSMC resulting in impaired response to GTN in the human forearm vessels. On the other hand, abnormal composition of low-density lipoprotein particles in the presence of hyperglycemia and hypertriglyceridemia may also contribute to impaired vascular function.[31] In support of these results, both plasma glucose and TC were found to be independent predictors for FMD in our study.

Another key mediator of vascular dysfunction in the presence of nephropathy is the accumulation of endogenous NO synthase inhibitor, asymmetric dimethylarginine.[32] Indeed, the accumulation of asymmetric dimethylarginine has been shown to correlate with carotid IMT in patients with end-stage renal disease.[33] Although we had not measured these potential mediators for vascular dysfunction in our subjects, our overall results provide a possible explanation for the increased cardiovascular risk in patients with renal insufficiency with both structural and functional components.

Study limitations

The majority of our patients with nephropathy were treated with insulin (77%), which may have beneficial effects on NO-mediated vascular function, and thus may attenuate the difference between DMN+ and DMN− patients. Nevertheless, most of the vasodilating actions of insulin occur in the microcirculation and are unlikely to be a major confounder[34] Although acute hyperglycemia might theoretically interfere with endogenous NO availability,[35,36] the overall evidence remain inconclusive.[37] On the other hand, the use of euglycemic-insulin clamp may induce problems such as pain which can interfere with local blood flow and is not always used in vascular studies of diabetic patients.[16,23,38] Indeed, in our study, we observed only minor differences in FMD, GTN-induced dilation, and IMT between the non-DM and DMN− subjects which might be in part due to their near optimal metabolic control and preserved renal function in contrast to patients with overt nephropathy. Since our study did not include patients with significant proteinuria (UAE > 300 mg/day) but preserved GFR, our results may not be applicable to this group of type II diabetic patients.

In conclusion, vascular defect in type II diabetic patients with overt nephropathy and renal insufficiency extends beyond the vascular endothelium and affects VSMC response to NO. Carotid IMT is also greatly increased in patients with diabetic nephropathy compared with those without. These functional and structural abnormalities may contribute to the markedly escalated cardiovascular risk in patients with diabetic renal disease.

MATERIALS AND METHODS

Patients and methods

The study was approved by the Chinese University of Hong Kong Clinical Research Ethics Committee. Twenty-six type II diabetic subjects with overt nephropathy and moderate renal insufficiency (DMN+) defined as 24 h UAE $\geqslant$300 mg/day on at least two occasions 6 weeks apart and plasma creatinine 130–260 μmol/l (patients with UAE $\geqslant$300 mg/day but plasma creatinine < 130 μmol/l were excluded) as well as 32 type II diabetic patients without nephropathy (non-DMN) defined as UAE < 300 mg/day on two occasions and plasma creatinine < 80 μmol/l in women and < 106 μmol/l in men were recruited from the Diabetes Centre of the Prince of Wales Hospital. A total of 52 age-matched volunteers (non-DM) who had no known medical history and fasting plasma glucose (< 5 mmol/l) were selected as control subjects. Type II diabetes was defined by the 1998 WHO criteria[39] and patients with acute and unprovoked ketotic presentation or who required insulin within 1 year of diagnosis were excluded.

All subjects arrived at the clinic after at least 10 h overnight fast without taking any medication on the study day. Informed written consent was obtained after the subjects were given detailed explanation about the study. Body weight and height were measured with subject wearing light clothing and without shoes. Waist circumference was taken as the mean of two readings of the minimum circumference between the umbilicus and xiphoid

process. Hip circumference was taken as the mean of two measurements of the maximum circumference around the buttock and the symphysis pubis. Supine blood pressure was taken using standard sphygmomanometer after at least 10 min rest. Korotkoff sound V was taken as the diastolic blood pressure. Fasting blood samples were taken for the measurement of fasting plasma glucose, glycated haemoglobin, plasma creatinine, and full lipid profiles including TC, high-density lipoprotein cholesterol, triglyceride, and calculated low-density lipoprotein cholesterol. Twenty-four urine collections were made to document 24-h UAE after exclusion of urinary tract infection in all subjects.

Assessment of IMT and endothelial function

All studies were performed in the morning. Subjects lay at rest for at least 10 min before the first scan. Artery scan of the longitudinal section of both common carotid arteries from the carotid bulb to at least 2 cm proximal to the carotid bulb were obtained using a 7-MHz array transducer interfaced to a standard ATL HDI 5000 system with artery intima clearly identified on the ultrasound image. IMT of the common carotid artery was measured at three random spots between 1 and 2 cm proximal to the carotid bulb on both sides. The mean of the six readings were taken. The intraobserver and interobserver variability were both 1.3%.

The brachial artery was located several centimetres above the elbow. Arterial scans were then obtained in longitudinal section using the same ultrasound machine. When a satisfactory transducer position was located, the skin was marked accordingly and the arm remained in the same position throughout the study. A resting scan was recorded for measurement of vessel diameter, and blood flow velocity was measured by the Doppler flow signal (velocity–time integral). Reactive hyperemia was produced by inflation of a pneumatic tourniquet placed on the forearm to 300 mm Hg for 5 min followed by rapid release. A second scan was obtained for measurement of FMD, starting 30 s before and until 5 min after deflation. Flow velocity during hyperemia was measured by Doppler velocimetry immediately following cuff deflation. The subjects rested for 10–15 min before another scan was obtained, which was followed by the administration of sublingual GTN, 400 μg and performance of a final scan 4 min later. All scans were recorded on supera-VHS videotape for later off-line analysis. The intraobserver and interobserver variability were 2 and 3.1%, respectively. The operator was blinded to the identity of the subject but not blinded to the timing of the examination.

Laboratory assay

Plasma glucose, TC, triglyceride, high-density lipoprotein cholesterol, and urinary creatinine:albumin concentrations were measured on a Hitachi 911 automated analyzer (Boehringer Mannheim, Mannheim, Germany). Low-density lipoprotein cholesterol was calculated by the Friedewald's equation.[40] Plasma creatinine concentration was measured on a Dimension AR system (Dade Behring, Deerfield, IL, USA). Glycated hemoglobin was measured by an automated ion-exchange chromatographic method (Bio-Rad Laboratory, Hercules, CA. Normal range 5.1–6.4%).

Data analysis

In all subjects, IMT was measured as described in the Materials and Methods section. The diameter of the brachial artery was measured twice by the same observer who did not know the clinical profiles of the subjects and the mean of the two readings were taken. The diameter of the artery during post-deflation period was measured every 30 s before and until 5 min after deflation to identify the diameter at maximal dilation for each subject. FMD and GTN-induced dilation, both expressed as percent changes in vessel diameter, were calculated from the observer's measurement using the average results of four cardiac cycles per condition.

Statistical methods

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 9.0) software. Results are expressed as mean $\pm$ s.d. Baseline characteristics of the diabetic patients and control subjects were compared using analysis of variance, as were FMD, GTN-induced dilation, and IMT of the three groups. The Modification of Diet in Renal Disease Study equation was used to estimate the GFR of all subjects.[41] Multiple regression analysis was used to evaluate correlations between IMT, endothelial function, clinical, metabolic, and renal parameters. As FMD- and GTN-induced dilation can be significantly affected by baseline brachial artery diameter, further analysis was performed using post-FMD and GTN-induced vascular dilation as independent variables with adjustment for baseline brachial artery diameter. FMD was the primary variable for analysis. All other P-values were adjusted for multiple comparisons. A two-tailed P-value of <0.05 was considered statistically significant.

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